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# Development of protein-detecting microarrays and related devices

Thomas Kodadek

There is great interest in the development of devices capable of monitoring the levels and post-translational modification states of hundreds or thousands of proteins simultaneously. One way to do this would be to create protein-detecting microarrays roughly akin to the DNA microarrays that are used for genome-wide expression studies. Two major challenges must be addressed before practical devices of this type become available. One is the development of high-throughput methods for the isolation of protein-binding compounds that will act as capture molecules in the array. The second is the optimization of methods that register binding of target proteins to the immobilized ligands in a sensitive and quantitative fashion. Progress in these areas, and some of the challenges remaining, are reviewed in this article.

The development of DNA microarrays has enabled massively parallel studies of gene expression [1,2]. This has sparked great interest in the development of devices such as protein-detecting microarrays (PDMs) to allow similar experiments to be done at the protein level [3–5] (Fig. 1).

Why do we need PDMs? DNA microarrays have shown promise in advanced medical diagnostics. More specifically, several groups have shown that when the gene expression patterns of normal and diseased tissues are compared at the whole genome level, patterns of expression characteristic of the

particular disease state can be observed [6–9]. For example, tissue samples from patients with malignant forms of prostate cancer display a recognizably different pattern of mRNA expression to tissue samples from patients with a milder form of the disease [10]. However, the technical demands of DNA microarray analysis make it poorly suited for use in standard clinical laboratories. A more attractive approach would be to monitor a few key proteins directly. These might be biomarkers identified by DNA microarray analysis. In this case, the PDM required would be relatively simple, containing only 5–10 features. A more radical approach would be to use PDMs with hundreds or thousands of features for the direct analysis of blood, sputum or urine samples. It is reasonable to suggest that the body would react in a specific way to a particular disease state and produce a distinct ‘biosignature’ in a complex data set, such as the levels of 500 proteins in the blood. Considerable effort will be required to validate this indirect approach to diagnostics. But, in the best case, one could imagine that in the future a single blood test could be used to diagnose most conditions.

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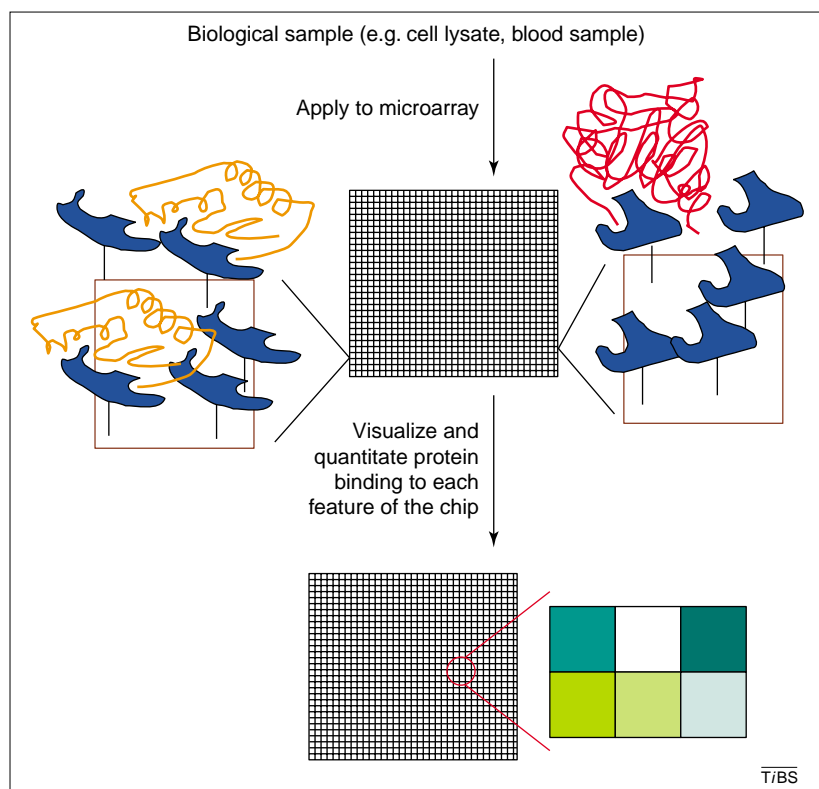


Fig. 1. A protein-detecting microarray (PDM). Each square in the grid represents a different feature of the array that would be impregnated with a particular protein ligand (blue shapes). When the sample is applied to the chip, each ligand will capture its target protein (orange and red coils in blow-up). Some other technique would then be used to quantitate the amount of binding of the target protein to each feature of the array. For example, the array could be probed with a collection of fluorescently labeled antibodies directed against the captured proteins. A scanner would then read the intensity of fluorescence (differently shaded green squares) at each spot, which would reflect the level of captured protein.

The motivation for the development of PDMs as basic research tools is different to that for their development for medical diagnostics. The utility of biosignatures is much lower as researchers will want to understand the molecular basis of cellular response to a particular genetic, physiological or environmental stimulus. DNA microarrays do a good job in this role already, but PDMs would allow for more accurate determination of protein levels [11] and, more importantly, could be designed to quantitate the presence of different splice variants or isoforms that differ in post-translational modifications. These events, to which DNA microarrays are largely or completely blind, often have pronounced effects on protein activities.

#### Current PDM technology

This field is in its infancy. PDMs that even approach the complexity of DNA microarrays do not exist. Indeed, the most readily available devices are 'virtual arrays'. For example, Luminex [12] and Illumina [13] market different forms of a virtual array in which an antibody raised against a protein of interest can be immobilized on a bead whose identity is encoded by a particular ratio of two covalently attached dyes. One hundred or so

uniquely encoded beads are added to a sample, resulting in capture of the proteins recognized by the immobilized antibodies. To quantitate the captured species, a sandwich assay is employed in which labeled antibodies that bind the captured proteins on a surface other than that recognized by the capture agent are added to the mix. The beads are then introduced into an instrument that reads the intensity of the fluorescence signal on each bead and also reads the identity of the bead by measuring the ratio of the dyes (Fig. 2). This technology is relatively fast and efficient, and can be adapted by researchers to monitor almost any set of proteins of interest, so long as good antibodies are available.

Closer to the 'true' PDM format is the ProteinChip® from CIPHERgen, which consists of an eight-feature array of antibodies (or other capture agents) embedded in a matrix suitable for ionization [14]. After application of the sample and removal of the unbound molecules (by washing), the retained proteins are analyzed by mass spectrometry. Proteolytic digestion of the bound species before ionization is also possible if peptide analysis is desired. A version of the chip is available in which each feature consists of a relatively non-specific capture agent such as an ion-exchange resin. Subsequent mass spectrometry analysis will therefore monitor the levels of many proteins captured at that feature. This technique should be particularly useful in biosignature analysis [14–16]. However, in the case where each feature consists of a specific capture agent such as an antibody, the relatively low throughput of this method will limit the number of proteins that can be monitored to <100.

Thus, although promising first-generation tools are available to researchers and diagnosticians, there remains the need to develop devices with much higher feature numbers and increased throughput. It seems probable that these systems will fall into two general categories. One will resemble more advanced versions of the CIPHERgen chip, in which modestly selective ligands capable of binding several different proteins will be arrayed, and the mixture of captured proteins will be deconvoluted and analyzed by mass spectrometry [17]. The other format will be a true array or virtual array device that uses large numbers of highly specific capture agents that target a single protein. The following discussion will focus on the major challenges that must be met in the development of the latter class of devices.

#### Antibodies: the natural choice

The heart of any array-type device for protein expression profiling will be the protein ligands that are used to create the array. The first wave of PDMs will almost certainly use antibodies because these are the most commonly used class of protein-binding molecule available [18,19]. Unfortunately, it is

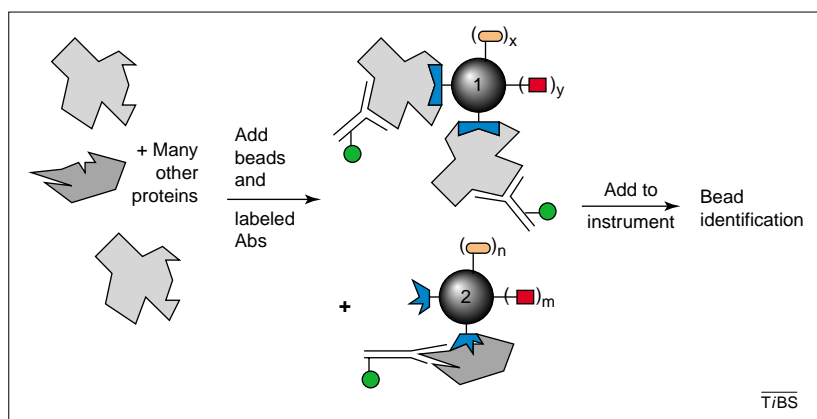


Fig. 2. An alternative format for the parallel detection of proteins. In this type of 'virtual array' each of many different beads displays a binding agent directed against a different protein target (blue shapes). Each different bead is color-coded by covalent linkage of two dyes (red and orange shapes) at a characteristic ratio. Only two different beads are shown for clarity. Upon application of the biological sample, the capture ligand binds the target protein. Then a mixture of secondary binding ligands [in this case antibodies (Abs)] conjugated to a fluorescent tag (green) is applied to the mixture of beads. The beads are then forced to pass single file through a detector where two lasers 'read' the ratio (n:m, x:y) of dyes and thus identify the bead, while the fluorescence intensity is read to quantitate the amount of labeled antibodies present (which will reflect the analyte level).

expensive and time-consuming to generate antibodies, particularly monoclonal antibodies, in the classical way. An alternative method is genetic immunization, which eliminates the requirement for a protein antigen. In this technique, a DNA fragment that expresses the polypeptide of interest is introduced by injection or gene gun into the animal [20]. The DNA is taken up by dendritic cells and makes its way to the nucleus. The encoded protein is expressed and an immune response is induced (both cellular and antibody). The titers of antibodies appear to be proportional to the expression level of the protein. The use of linear expression elements (PCR products in which a promoter and terminator flank the gene) has demonstrated that effective genetic antigens can be constructed without the need for cloning [21], making this a potentially high-throughput technique.

To do away with the need for large numbers of animals, libraries can be searched for single-chain antibodies that bind the protein of interest. Phage M13 is the most common display vector [22], but yeast, bacteria and other viruses have also been used [23]. The advantage of this approach is that once a single-chain antibody is isolated in a display experiment, one has a monoclonal source of that molecule. Unfortunately, the equilibrium dissociation constants ( $K_D$ s) of antigen complexed to antibodies isolated from libraries are generally not as low (often in the  $10^{-7}$  M range) as those from complexes using classically produced antibodies ( $10^{-9}$  M). The notable successes in this area have come from groups that have made very large libraries ( $\geq 10^9$ ), from which nanomolar binders can be isolated [24]. The chances of finding rare, high-affinity binders in a library increase with the size of the library (see following text).

#### Scaffolded peptides as antibody substitutes

An alternative approach is to search libraries of peptides displayed on the surface of a protein. The idea is that restricting the degrees of freedom of a peptide by incorporating it into a surface-exposed protein loop could reduce the entropic cost of binding to a target protein, resulting in higher affinity. Thioredoxin is a small, stable protein with a surface loop that will tolerate a great deal of sequence variation. Brent and co-workers replaced the native loop sequence of this protein with a 20 residue combinatorial peptide library, and expressed these proteins, along with the protein target of interest, in yeast as fusions that could be used in a two-hybrid screen [25,26]. Thioredoxin derivatives that bind their target with  $K_D$ s in the  $10^{-7}$ – $10^{-8}$  M range were obtained. Of great interest in this area is the development of smaller display scaffolds that are accessible synthetically. For example, Schepartz and colleagues have recently shown that the 33-residue avian pancreatic polypeptide (aPP) is useful for this purpose [27].

#### Simple peptides, peptidomimetic compounds and small molecules

Peptides are attractive candidates for ligands in PDM applications because they combine advantages of small molecules and proteins. Large, diverse libraries can be made either biologically or synthetically [28,29], and the 'hits' obtained in protein-binding screens can be made synthetically in large quantities. However, the affinities of peptides isolated from libraries are generally too low for PDM applications. The situation is even worse than that for antibody and protein aptamer libraries (an aptamer is defined as a specific protein-binding molecule). For peptides, a good result is a peptide–protein complex with a  $K_D$  of  $\sim 10^{-6}$  M. However, protein-binding peptides might be useful as pieces of more elaborate multivalent protein ligands (see following text).

Peptide-like oligomers [30] such as peptoids [31] have certain advantages over peptides. They are impervious to proteases and their synthesis can be simpler and cheaper than that of peptides [32], particularly if one considers the use of functionality that is not found in the 20 common amino acids. However, peptidomimetic compounds share the Achilles heel of peptides in that they rarely bind with sufficient affinity to be of use in PDM devices. This is also true of non-peptidic, more drug-like molecules, libraries of which are also more difficult to make, despite impressive recent advances [33].

#### Nucleic acid ligands

There have been several reports of the isolation of high-affinity protein-binding nucleic acids selected from libraries [34,35]. Indeed, given the ease of creating, amplifying and evolving libraries of nucleic acids [36], it is probable that this class of molecules will be a rich source of high-affinity protein-binding

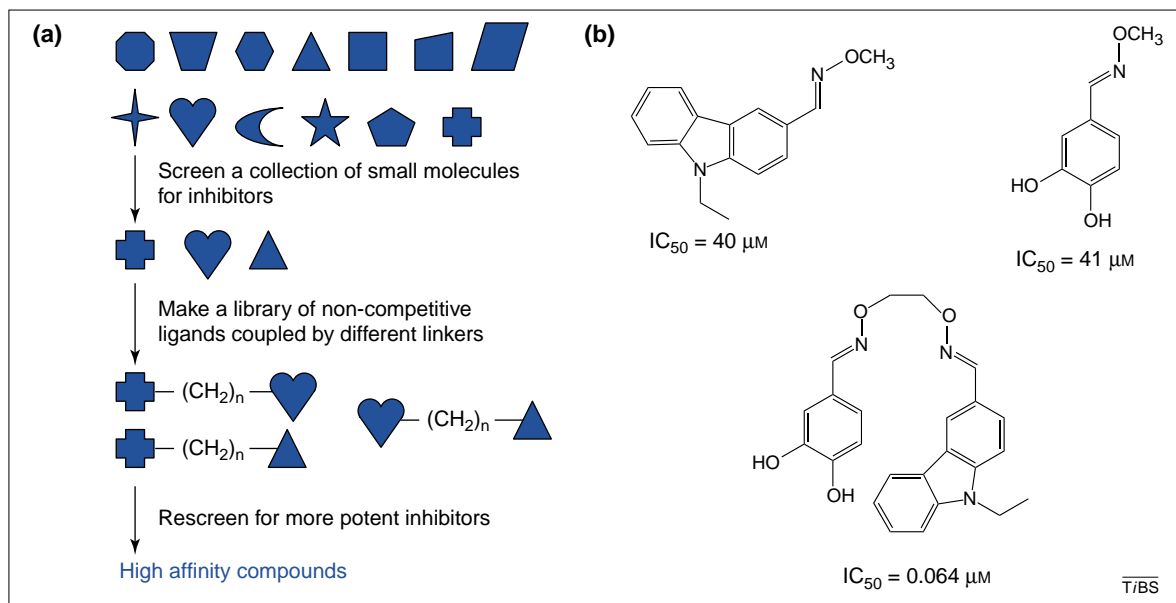


Fig. 3. (a) A method of discovering high-affinity enzyme inhibitors reported by Ellman and co-workers [42]. First, a collection of compounds is screened at high concentrations for weak inhibitors of some enzymatic activity (in the case of the Ellman study, inhibition of cSrc-catalyzed peptide phosphorylation). The compounds that do not compete with one another are then identified and a library of chimeric compounds is made with linkers of different length. This library is then screened at much lower concentrations to identify potent inhibitors. The same protocol could potentially be applied to the discovery of ligands for proteins that are not enzymes or are of unknown function if appropriate binding assays were substituted for the inhibition assays used in this study. (b) Top: low affinity inhibitors of c-Src identified in a primary screen. Bottom: chimeric compound created by joining the two low affinity inhibitors; this chimera displays potent inhibition of c-Src [42].

ligands. Of course, as with any class of molecules, nucleic acids have their weaknesses. Given the negatively charged character of nucleic acids, one might predict that they would work best against protein targets that are natural nucleic acid-binding proteins or at least have positively charged surface patches. Another issue is that most of the work done in this area has focused on RNA molecules. The chemical synthesis of RNA is not as simple as that of DNA, so large-scale production would be more problematic.

#### The road to higher-affinity ligands: size matters

The likelihood that a given compound in a random collection will be a high-affinity ligand for a given protein is low, and these molecules will usually be present only in large libraries. For example, streptavidin ligands with  $K_D$ s as low as 5 nM were isolated from a library of  $\sim 10^{13}$  88-residue peptides [37]. This study used the powerful mRNA display technology developed by Roberts and Szostak [38], in which the library is made by *in vitro* transcription and translation. The translation reaction is conducted such that the encoding mRNA and the translated peptide are held together covalently via a puromycin linker, essentially labeling the peptide library with a tag that can be amplified by PCR (for a conceptually similar approach called ribosome

display, see [39]). The library is incubated with an immobilized target protein, the unbound molecules are washed away and the mRNAs connected to the binding peptides are amplified by RT-PCR. This enriched pool is then used as the input in further rounds of selection. The protocol is not unlike the more commonly used phage display technique, in which the virus provides a link between the peptide and the encoding DNA, as well as allowing for amplification of rare binders. The major difference is that much larger libraries can be used in the *in vitro* mRNA or ribosome display methods because there is no limitation imposed by the efficiency of transformation. The selection of RNA aptamers is also a completely *in vitro* protocol and, in fact, even larger libraries can be screened in this case ( $10^{15}$  molecules). Mutagenesis can be incorporated into the amplification step to allow for 'evolution' through rounds of selection [40], as can also be done in the mRNA and ribosome display protocols.

#### Chimeric binding agents derived from two low-affinity ligands

Although powerful, the brute force approach of using extremely large libraries is limited to *in vitro* screens of biomolecules. It would be impossible, for example, to conduct a solid phase synthesis of  $10^{13}$  different drug-like molecules or peptidomimetic compounds. Libraries containing  $10^5$ – $10^6$  compounds are considered large in the synthetic arena. An attractive alternative strategy is to link two or more modest-affinity ligands. Given the appropriate linker, such chimeric compounds should exhibit affinities that approach the products of the affinities for the two individual ligands.

One such approach relied on structure-based linker design. Nuclear magnetic resonance was used to screen several commercially available small molecules for binding to FKBP (FK506-binding protein). A structure was then solved for each small



molecule–FKBP complex uncovered in the initial screen. These data both identified ligand pairs that bound different surfaces of the protein (i.e. did not compete with one another) and enabled the design of linkers that were predicted to allow for cooperative binding of the two molecules. Using this method, a simple chimeric molecule that binds FKBP with a  $K_D$  of 19 nM was obtained [41].

The second approach of this type was described by Ellman and colleagues [42]. In this case, low affinity inhibitors of the cSrc kinase were identified from a collection of aldehyde-derived oxime derivatives (Fig. 3). The initial hits were then tested in combination to determine which molecules appeared to bind non-competitively. A combinatorial strategy was then used to create a small library of chimeric molecules containing dioxime linkers of different lengths. This collection of compounds was rescreened under more dilute conditions for more potent inhibitors. An inhibitor with a  $K_i$  (inhibition constant) of ~60 nM was obtained.

These studies provide important precedents for the feasibility of transforming low-affinity ligands into high-affinity species via a 'pincer' strategy. There is no reason that it could not be applied to peptides or any other type of modest-affinity protein-binding compound. The trick will be to further streamline the determination of which monomeric ligands bind non-competitively and to accelerate the discovery of optimal linkers in the absence of structural information.

#### Quantitation of ligand–protein binding events

Another important aspect of developing practical PDMs is to quantitate binding of analyte proteins to each feature of the array. Broadly speaking, the candidate solutions can be split into three groups: (1) sandwich assays, (2) direct measurement of the bound protein by mass spectroscopy or some other analytical technique, and (3) the use of capture agents and/or array surfaces that change their chemical or physical properties upon protein binding.

Sandwich protocols were discussed above in the context of Luminex-type virtual arrays. The fact that a signal is registered only if two independent binding events occur provides a major boost in specificity. The downside is that this approach doubles the number of ligands that must be isolated for each protein of interest.

At present, a lot of activity is focused on the development of 'smart materials' – binding molecules and/or array surfaces whose properties change in some measurable way upon protein binding; for example, multi-site surface plasmon resonance (SPR) chips [43]. One idea is to mount a ligand on a gold-coated silicon nitride microcantilever. Binding of a protein to the ligand generates sufficient force to deflect the cantilever and generate motion, which can be detected optically. For example, when a polyclonal antibody

raised against prostate-specific antigen (PSA) was immobilized on the cantilever via a gold–thiol interaction, PSA concentrations ranging from 0.2 ng ml<sup>-1</sup> to 60 µg ml<sup>-1</sup> could be detected in the presence of a large excess of serum albumin [44]. In the DNA-detection arena, alteration of the ability of an electroactive ligand to conduct charge has been used effectively to follow analyte binding [45].

Some investigators have begun to explore smart ligands – molecules that somehow report directly the fact that they have bound the protein target. For example, using *in vitro* selection and evolution methods, Breaker and colleagues [46] isolated ribozymes capable of self-cleavage in response to binding of a specific analyte such as a small molecule or a protein. One end of the ribozyme was affixed to a gold-coated surface via incorporation of a sulfur atom into the RNA. The ribozyme also had a radiolabel that was positioned in such a way that the label was lost upon analyte-dependent self-cleavage. Seven different ribozymes, each responsive to a different molecule, were arrayed and exposed to differing concentrations of the different analytes. It was found that the loss of radioactivity from each spot was proportional to the concentration of the corresponding target molecule. However, binding-dependent catalytic activity is a difficult thing to achieve and this strategy might be restricted to nucleic acid capture agents.

#### Detection of post-translational modifications and the complications of protein–protein interactions

As mentioned above, one of the primary reasons for developing PDMs is to have an analytical tool sensitive to the post-translational modification (ptm) state of a protein. However, a capture agent that is insensitive to the ptm state of the target protein mounted on a smart surface will be blind to these effects. This is the Achilles heel of the otherwise attractive 'smart surface' approach to detecting ligand–protein interactions. A distinct and highly specific capture agent will have to be generated for each post-translationally modified form of interest, an impossible task on a proteome-wide scale.

Protein–protein interactions will also be a complicating factor for certain types of detection strategy. For example, SPR provides a signal that is proportional to the mass of the species that associates with the surface-bound ligand. In many cases, the target protein will be part of a multiprotein complex, but the identities of its partners will often be unknown. This will give rise to major uncertainties in how to relate the intensity of the signal to a concentration of the target protein. There are several other issues relevant to this point that have been discussed previously [4].

The ptm and protein interaction issues are more easily accommodated with other detection protocols. If mass spectrometry were to be used, chemically different forms of the protein could, in principle, be characterized in the mass spectrum; however, in

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reality, things are not so straightforward. In addition, the existence of protein–protein interactions should not constitute a major problem if the proteins are ionized under conditions that disrupt non-covalent interactions, leading to separate peaks for each polypeptide or fragment derived from them. Protein–protein interactions are also not a major problem in sandwich assays, because the signal is generated by binding of the labeled secondary reagent to the specific ligand-bound target protein of interest. The sandwich approach could also be adapted to provide information on the ptm state of the protein target. For example, if the capture agent was ptm-insensitive (but protein-specific) and the secondary reagent was targeted to a particular ptm (but was protein non-specific), then the signal would reflect only a particular ptm state of the protein of

interest. An anti-phosphotyrosine antibody is a good example of this type of secondary reagent.

For these reasons, there will probably be a bifurcation in the types of protein-detecting arrays that emerge from the next several years of work. One type will be arrays of modest size comprising a smart surface derivatized with a limited number of highly sophisticated capture ligands that recognize only a particular post-translationally modified form of a given protein. These optimized devices will be used in cases where the few proteins detected have been validated as high value targets, perhaps for diagnostic purposes. The other major class of devices will be much larger arrays of ptm-insensitive ligands combined with detection strategies such as mass spectrometry, or the type of sandwich assays discussed above. These will be the research tools for large-scale protein expression profiling experiments.

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